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α-METHYLGLUCOSIDE TRANSPORT BY THE GILL OF THE OYSTER *OSTREA EDULIS*[1]

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Abstract

The glucose transport system in the gill of *Ostrea edulis* was studied using α-methyl[U-^{14}C] glucoside. The same mobile carrier transported α-methylglucoside (α-MG) and D-glucose in the isolated gill. Unlike D-glucose α-MG was not appreciably metabolized to CO_2. It was hypothesized that the mobile carrier is specific for the hydroxyl group at the C_3 position of D-glucose. Ouabain (0.1 m*M*) did not reduce α-MG uptake, thus suggesting that Na^+ transport was either via a ouabain insensitive pump or a ouabain sensitive pump inaccessible to ouabain dissolved in seawater, the external medium. Compounds capable of collapsing the transmembrane electrical potential reduced α-MG uptake. Naphthalene, a compound known to hyperpolarize muscle cells, stimulated α-MG uptake. Inner leaks via the mobile carrier were determined to be much greater than outer leaks. The results indicate that the glucose transport system in the oyster gill has many similarities to Na^+-dependent transport of glucose in mammalian small intestines and proximal renal tubules. Naphthalene stimulation of α-MG uptake is consistent with a previous study demonstrating naphthalene stimulation of glucose metabolism in gill tissue.

Introduction

The uptake of simple sugars by the bivalve gill was described by Péquignat (1973) and Bamford and Gingles (1974), who showed that D-glucose and D-galactose were rapidly taken up by the isolated gill of *Crassostrea gigas.* However, 3-0-methyl-D-glucose (3-MG) was not taken up. Glucose uptake depended on the external Na^+ concentration. Thus, Bamford and Gingles (1974) concluded that the mechanism of transport was probably a Na^+ -glucose co-transport system.

The glucose transport system in the gill removes dissolved glucose from seawater. Sources of naturally-occurring glucose include plant and animal excretions and decomposition (Vaccaro and Jannasch, 1966). Active uptake of dissolved organic molecules may provide a significant supplement to the nutrition of bivalves (Wright and Stephens, 1978). Additionally, active reabsorption may serve to counteract the leakage of metabolically important substrates from the intra and intercellular space.

The study of the glucose transport system is of interest from both a physiological and toxicological perspective. Membrane bound transport systems are important

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Abbreviations: 3-MG, 3-0-methyl-D-glucose; α-MG α-methylglucoside; DNP, dinitrophenol; CCCP, carbonylcyanide-M-chlorophenylhydrazone; PCP, pentachlorophenol; $[MG]_{in}$ $[MG]_{out}^{-1}$, concentration ratio of α-MG in tissue and seawater; K_i, inhibitor constant; R_f, mobility relative to the solvent front.

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control points of metabolism (Elbrink and Bihler, 1975; Weiner, 1979). The first site of interaction between chemical toxicants and living tissue is the cell membrane (Marchesi, 1978; Pritchard, 1979). Thus, membrane bound transport systems may serve as useful probes for studying the metabolic effects of lipophilic toxicants such as naphthalene. The glucose transport system is especially suited for such study since nonmetabolized analogs of glucose (*i.e.* methylglucosides) are available which allows the study of transport in isolation from subsequent metabolic transformation.

The present study more clearly defines the mechanism of glucose transport in the isolated gill of oysters, using the European flat oyster, *Ostrea edulis*, as a representative species. The specific objectives were: (1) identify a non-metabolized transport analog, (2) determine the effects of known metabolic inhibitors and naphthalene on transport, and (3) determine the effect of inhibitors on leakage.

Materials and Methods

All analytical chemicals were at least reagent grade. D-[U-^{14}C]glucose (274 mCi mmol^{-1}), [^{3}H]inulin (1.9 Ci mmol^{-1}), [1-^{14}C]naphthalene (36 mCi mmol^{-1}), and α-methyl[U-^{14}C]glucoside (α-MG) (184 mCi mmol^{-1}) were obtained from the Amersham Corporation (Arlington Heights, Illinois). Dinitrophenol (DNP), D-glucose, α-methylglucoside (α-MG), and ouabain were obtained from Sigma Chemical Co.; carbonylcyanide-M-chlorophenylhydrazone (CCCP) from Aldrich; iodoacetate (Na^+-salt) and pentachlorophenol (PCP) from Baker Chemicals. Curve fitting was accomplished by regression analysis (Neter and Wasserman, 1974). The mathematical models were transformed to the appropriate linear form and then the best fit judged by visual inspection and the magnitude of the coefficient of determination, r^2.

Pre-conditioning of oysters

Oysters (*O. edulis*) obtained as cultchless spat from International Shellfish Enterprises, Inc., Moss Landing, California, were held in flowing seawater at the Oregon State University Marine Science Center at Newport, Oregon. When approximately 1 year old, they were transferred to a closed holding aquarium (Instant Ocean) and maintained at 28.5 ‰ and 15°C with no apparent source of nutrition for 6–10 days. All experiments were conducted at 15°C in air-saturated Millipore-filtered (0.22 μm) seawater. The gill and overlying mantle were removed immediately before each experiment. The isolated gill preparation was composed of all four intact demibranchs (ascending and descending lamellae) and the associated mantle tissue.

Metabolism of α-methylglucoside (α-MG)

The gill tissues from 8 oysters were placed into separate incubation vessels containing 40 ml of filtered seawater, 200 ppm streptomycin, 100 ppm chloramphenicol, and 300 ppm penicillin each. D-[U-^{14}C]glucose (2 μCi) was added to four of the vessels and α-[U-^{14}C]MG (2 μCi) added to the remaining four. Each vessel was sealed with a Teflon gasket and aerated; the effluent air from each vessel was bubbled through a CO_2 trap containing 5 ml of Oxisorb-CO_2 (New England Nuclear, Boston, Massachusetts). After 1 h the tissues were removed and digested in NCS tissue solubilizer (Amersham Corporation), the vessels were resealed, 1 ml of concentrated sulfuric acid was injected into each vessel via a septum, and aeration was resumed for 1 h. The contents of the CO_2 traps, samples of the

seawater, and the tissue digests were assayed by standard liquid scintillation counting procedures on a Packard Tri-Carb LSC.

Uptake of α-MG

A tissue holder was constructed that held up to 50 tissues, each suspended on a separate stainless steel stylet. Groups of 3–10 tissues were suspended in this way in incubation vessels containing 200 ml of filtered seawater and [^{3}H]inulin. The ratio of seawater to tissue was always greater than 100:1. After 30–45 min of incubation in [^{3}H]inulin, the tissues were transferred simultaneously, on the tissue holders, to a fresh set of incubation vessels containing 200 ml of filtered seawater, [^{3}H]inulin, and α-[U-^{14}C]MG. The incubation vessels and tissue holders were maintained on a gyrotary shaker at 125 rpm. The system was designed so that as few as three tissues, or all the tissues (50) could be removed simultaneously and weighed wet, freeze-dried, or transferred to individual digestion tubes containing 1 *N* sodium hydroxide (NaOH). Samples of seawater were assayed for radioactivity at the beginning and end of each experiment to monitor constancy of the substrate concentration. Tissues were digested in 1 *N* NaOH at 50°C for 2–4 h, cooled, the volume adjusted, and then a sample counted in PCS (Amersham Corporation) scintillation fluor. A second sample was analyzed for total protein (Lowry *et al.*, 1951). Standard dual-channel counting procedures were used to assay for ^{3}H and ^{14}C. The accumulation of α-MG in the tissue was corrected for the inulin (extracellular) space (Schultz *et al.*, 1966).

Using these procedures, tissues were incubated for different lengths of time at the same substrate concentration or for fixed lengths of time at different substrate concentrations.

Transport product of α-MG

Tissues were incubated in α-[U-^{14}C]MG for up to 30 min. When removed, the tissues were quickly rinsed, frozen on an aluminum sheet (−70°C), and freeze-dried. The dry tissue was weighed, pulverized after cooling with liquid N_2, and homogenized in cold (4°C) water. The cold homogenizing tube was plunged into a boiling water bath and the contents heated rapidly to 91°C. The tube was removed, cooled slowly, and centrifuged at 16,000 × *g*. The supernatant was freeze-dried. For thin layer chromatography, the freeze-dried supernatant was dissolved in a small volume of water. A 20 μl sample was spotted on a silica-gel thin layer plate and developed in chloroform:methanol:water (60:70:26) (Kaback, 1968). The developed plates were scanned with a Varian Aerograph Radiochromatogram Scanner.

Effect of inhibitors and naphthalene on α-MG uptake

Inhibitors of Na^+K^+ transport (ouabain), glycolysis (iodoacetate), protonophores (DNP, CCCP, PCP), phloridzin (phloretin-2′-β-D-glucoside), and D-glucose were tested for their effect on α-MG uptake. Naphthalene, a compound known to hyperpolarize muscle cells (Nelson and Mangel, 1979), and stimulate glucose metabolism (Riley and Mix, 1981), was also tested. Ouabain, indoacetate, DNP, CCCP, PCP and naphthalene were dissolved in 80% ethyl alcohol before being diluted in the filtered seawater. Phloridzin and D-glucose were dissolved directly into the seawater. Fifty μl of ethyl alcohol was added to all treatments and controls.

The isolated gill tissues were pre-incubated for 30 min in 200 ml of filtered seawater containing either one of the inhibitors or naphthalene; and then transferred to a fresh set of incubation vessels containing 200 ml of filtered seawater, inhibitor or naphthalene, and 50 μM α-[U-^{14}C]MG. Tissues were then incubated for an additional 30 min. The incubation vessels were maintained on a gyrotory shaker at 125 rpm.

Leakage of α-MG

Tissues were incubated as described in the section on uptake of α-MG. However, after loading with α-[U-^{14}C]MG, 30–45 min, the tissues were transferred to tubes containing 4–8 ml of filtered seawater containing either no additives, 0.5 m*M* DNP, 0.25 m*M* CCCP, 0.25 m*M* PCP, 0.20 m*M* phloridzin or 0.20 m*M* D-glucose. The tubes were mixed (125 rpm) for 30 min. The tissues were then digested and a sample of the seawater counted as before. The effect of naphthalene on leakage was also determined. The seawater media used for loading tissues with α-[U-^{14}C]MG was made 1 μg ml^{-1} with naphthalene and then the tissues treated as described above.

The oysters used in this study were small. The wet weight of the gills was 0.150 g $\pm$ 0.076 (mean ($\bar{X}$) $\pm$ standard deviation (SD), n = 149). The inulin space was 0.415 ml (g wet wt)$^{-1}$ $\pm$ 0.102 ($\bar{X}$ $\pm$ SD, n = 80). This value compared reasonably well with the volume of the extracellular space in *Mytilus californianus* (46% of the whole body) (Thompson *et al.*, 1978).

Results

Metabolism of α-MG

Gill tissue did not readily metabolize α-MG. Fifteen times as much glucose-carbon was converted to CO_2 as α-MG-carbon. After 1 h, 17.69% (SD = $\pm$ 6.09, n = 4) and 15.96% (SD = $\pm$ 2.88, n = 4) of the radioactivity from D-glucose and α-MG, respectively, were in the tissue digests and CO_2 traps. The remaining radioactivity was in the seawater. The percent of the accumulated dose (digests + CO_2) disposed to CO_2 was 14.49% (SD = $\pm$ 4.98, n = 4) for glucose and 0.97% (SD = $\pm$ 0.44, n = 4) for α-MG. The α-MG-carbon metabolized to CO_2 could partially be attributed to residual glucose contamination as a result of the synthesis of α-MG from glucose. α-MG was determined by the manufacturer to be 99% pure.

Uptake of α-MG

The α-MG was more concentrated in tissue than in the seawater. Uptake was linear over 90 min (Fig. 1a). The rate of α-MG uptake followed typical saturation kinetics, and adding D-glucose (50 μM) reduced the rate of uptake in a manner indicating competitive inhibition (Fig. 1b). Without glucose present the apparent K_t and V_{max} were estimated as 50.7 μM and 0.4216 μmol g-protein$^{-1}\cdot$min^{-1}, respectively (Fig. 1c). In the presence of 50 μM glucose, the K_t and V_{max} were 148.5 μM and 0.4700 μmol g-protein$^{-1}\cdot$min^{-1}, respectively. Glucose (50 μM) increased the apparent K_t by a factor of 3, but had little effect on V_{max}, thus indicating that α-MG and D-glucose uptake were mediated by the same transport system.

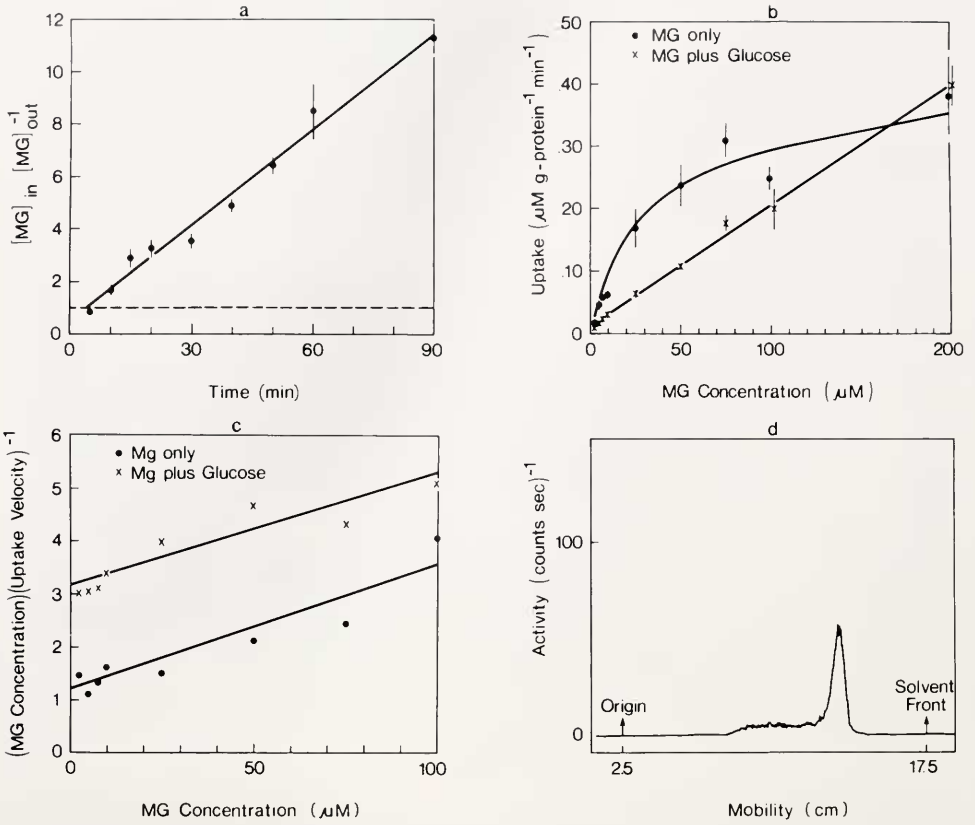

FIGURE 1. (a) The time course of α-MG uptake plotted as the ratio of α-MG in the tissue ($[MG]_{in}$) and α-MG in the seawater ($[MG]_{out}$). The concentration in the seawater was 20 μM. Each datum is the $\bar{X} \pm SE$, n = 4. The dotted line represents the α-MG uptake expected by diffusion alone. (b) The rate of α-MG uptake as a function of increasing substrate concentration with and without 50 μM D-glucose added. Each datum is the $\bar{X} \pm SE$, n = 3. (c) Linear transformation, Hanes-Woolf plot (Segel, 1976), of the data from (b). Slopes represent $1/V_{max}$ (entry max) and intercepts K_t/V_{max}. (d) Typical radiochromatogram scan of the aqueous extract from α-Mg loaded tissues. The R_f of the single radioactive peak is the same as authentic α-MG ($\simeq$0.66).

Transport product of α-MG

Radiochromatogram scans indicated that all accumulated radioactivity was recovered as a single, well resolved peak (Fig. 1d) whose mobility was the same as the original α-MG prior to accumulation.

Effect of inhibitors and naphthalene on α-MG accumulation

Protonophores were the only metabolic inhibitors that significantly ($P < 0.05$) inhibited α-MG uptake (Fig. 2, 3). Concentrations of CCCP and PCP as low as 2.5 μM reduced uptake by 50%. However, even in the presence of 250 μM CCCP or PCP, uptake was greater than would be expected from diffusion alone. For diffusion alone, $[MG]_{in}\,[MG]_{out}^{-1}$ should equal 1.0.

Phloridzin was the only inhibitor that reduced α-MG uptake to less than ex-

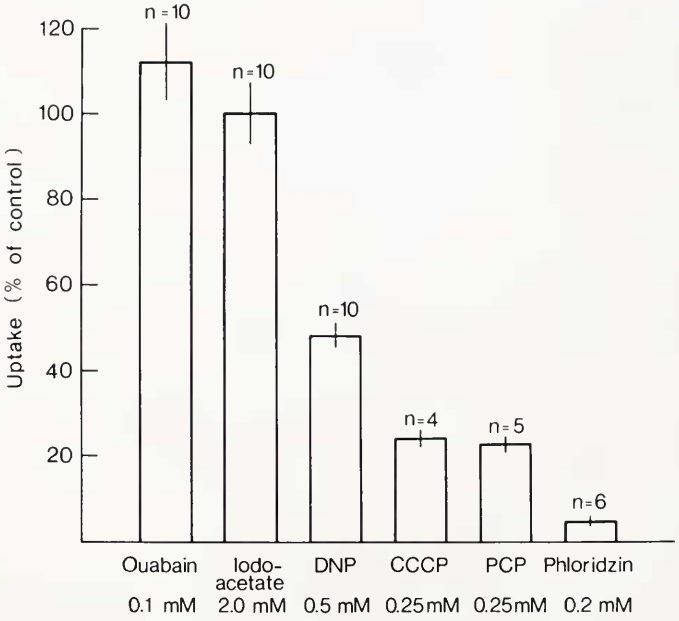

FIGURE 2. The effect of metabolic inhibitors and the competitive inhibitor, phloridzin, on α-MG uptake. The total incubation time with the inhibitor was 60 min, 30 min of which was with 50 μM α-MG. Values expressed as a percent (±SE) of the mean concentration ratio for controls, uncorrected for diffusion.

pected based on diffusion alone. The calculated uptake attributable to diffusion was 13.6% ± 2.9 ($\bar{X}$ ± SD, n = 5) of the total α-MG uptake. Uptake in the presence of 200 μM phloridzin was 4.5% ± 0.6 ($\bar{X}$ ± SD, n = 6) of the uptake of controls (Fig. 2); significantly less than expected based on diffusion alone.

Naphthalene-treated gills took up significantly greater amounts of α-MG than controls (Table I). Uptake of α-MG ranged from 132% to 141% of controls.

Leakage of α-MG

All inhibitors of α-MG uptake significantly ($P < 0.05$) increased leakage of ^{14}C-label from α-MG-loaded gills (Fig. 4). Phloridzin was least effective and D-glucose the most effective at stimulating leakage.

Naphthalene treatment slightly decreased leakage, but the effect was not significant ($P > 0.05$). Leakage of α-MG after 30 min was 1.26% ± 0.55 (n = 10) and 1.66% ± 0.63 (n = 9) for naphthalene-treated and control-treated gills, respectively.

DISCUSSION

D-glucose and α-MG were transported by the same carrier in the gill of *O. edulis*. Once accumulated, D-glucose was extensively metabolized to CO_2, while α-MG was not. Thus, α-MG is a transport analog of D-glucose not metabolized via the classical aerobic pathway of glucose metabolism. It was taken up against its chemical potential gradient, and preliminary evidence (one-dimensional TLC)

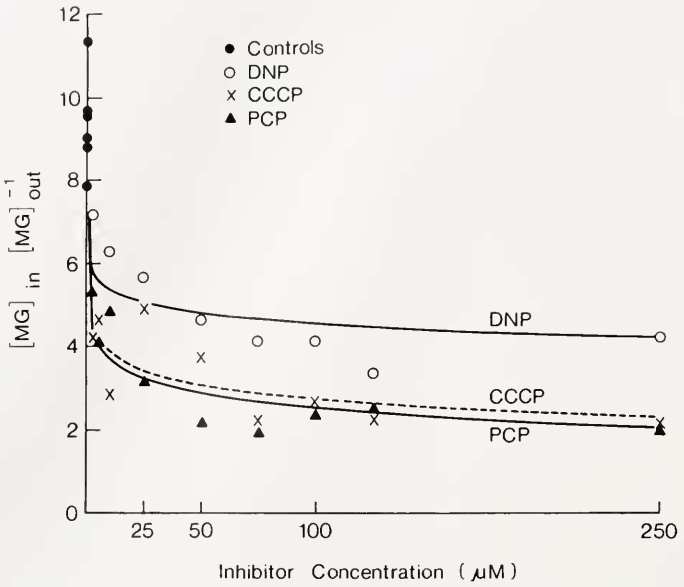

FIGURE 3. The effect of protonophore (DNP, CCCP, PCP) concentration on α-MG uptake. The total incubation time with the inhibitor was 60 min, 30 min of which was with 50 μM α-MG. The ratio of α-MG in the tissue ($[MG]_{in}$) to that in the seawater ($[MG]_{out}$) for the controls was 9.34 ± 0.48 ($\bar{X}$ ± SE, n = 6).

indicated that it accumulated chemically unaltered. In mammalian tissues, only the epithelial cells of the small intestine and kidney proximal tubules take up sugars against their chemical potential gradient (Crane, 1977), with the substrate specificity varying between tissues. The structural requirements of the oyster gill are similar to the rabbit renal brush border (Aronson and Sacktor, 1975). The glucose carrier in the oyster gill is specific for the hydroxyl group at the C_3 position on the glucose molecule, but not for the hydroxyl at C_1. The orientation of the hydroxyl at C_4 is not critical. These structural requirements were deduced from the fact that

TABLE I

Effect of 1 μg ml^{-1} (initial concentration) naphthalene on α-MG uptake by isolated gills in three separate experiments (1 to 3). Values calculated as $[MG]_{in}$ $[MG]_{out}^{-1}$ ± 95% C. I.; number of tissues in parentheses. Ct: control-treated; Nt: Naphthalene-treated. The total incubation time with naphthalene was 60 min, 30 min of which was with 50 μM α-MG.

Experiment	Ct	Nt
1*	4.99 ± 0.83 (9)	6.56 ± 1.06 (9)
2**	5.36 ± 0.48 (10)	7.53 ± 0.82 (10)
3**	4.93 ± 0.53 (10)	6.96 ± 0.70 (10)

* $P < 0.025$; Student's t test.
**$P < 0.001$; Student's t test.

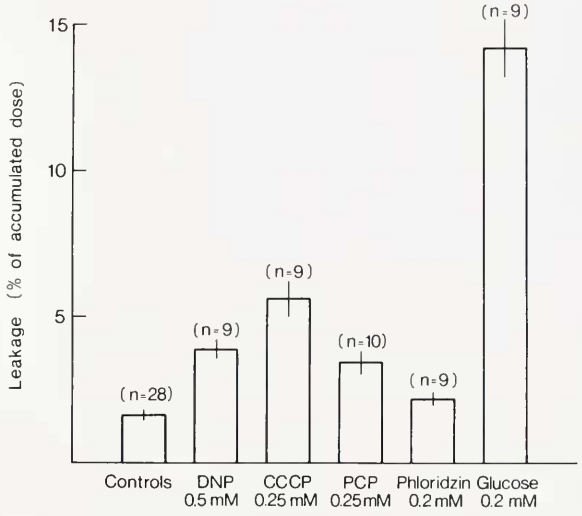

FIGURE 4. The effect of protonophores (DNP, CCCP, PCP) and competitive inhibitors (phloridzin, D-glucose) on α-MG leakage. Values expressed as a percent (±SE) of the total α-MG accumulated in the tissue after 30 min incubation in α-MG followed by 30 min leakage in the presence of the inhibitor.

D-glucose and D-galactose, but not 3-MG were taken up by the gill of *C. gigas* (Bamford and Gingles, 1974), and α-MG was taken up by *O. edulis*.

Comparison of kinetic data from different studies is of questionable validity since the physiological state of the tissue greatly influences the kinetics of transport. Wright (1979) demonstrated that isolation of gill filaments from *Mytilus californianus* reduced activity of the lateral cilia and subsequently increased apparent Michaelis constants (K_t) for glycine transport. However, study of uptake using isolated tissues is valid for studying mechanisms of transport and possibly for determining maximum transport velocities.

The fact that α-MG uptake was not reduced in the presence of ouabain suggests that either Na^+ transport out of the intracellular space is dependent on a ouabain insensitive pump, or that the ouabain sensitive Na^+ pump is inaccessible to ouabain dissolved in the external seawater medium. Sodium-gradient-coupled sugar and amino acid transport is well documented in mammalian tissues (Crane, 1977) and the gill epithelia of bivalves (Bamford and Gingles, 1974; Wright and Stephens, 1977). Typically, the basic model of the Na^+ gradient hypothesis requires 2 components: a mobile Na^+ plus substrate carrier and an energized Na^+ pump (Crane, 1977). In mammalian small intestines, the Na^+-coupled transport of glucose depends on the transmembrane electrochemical potential gradient, maintained by the Na^+ pump, which is the sum of the transmembrane chemical potential gradient (concentration ratio of Na^+ across the cell membrane) and the electrical membrane potential (Crane, 1977). Compounds which inhibit the Na^+ pump or abolish the membrane potential will also inhibit glucose accumulation. Therefore, it was surprising that ouabain, a compound known to inhibit the Na^+ pump in the plasma membrane, had no inhibitory effect on α-MG uptake by the oyster gill. Stewart and Bamford (1975) found that uptake of alanine by the gill of *Mya arenaria* was relatively insensitive to ouabain (2mM) unless tissue strips were first pre-incubated for 60 min. Since the gill-mantle complex of estuarine clams is known to be rich

in Na^+K^+ ATPase activity (Saintsing and Towle, 1978), insensitivity to ouabain suggests that the Na^+ pump is inaccessible to ouabain dissolved in seawater. Silva *et al.* (1977) demonstrated in the American eel, *Anquilla rostrata*, that Na^+K^+ ATPase in gill epithelia pumps Na^+ into the extracellular fluid rather than external seawater. Ouabain circulating in the blood of the eel effectively inhibited Na^+K^+ ATPase in the gills while ouabain in seawater was much less effective (Silva *et al.*, 1977). Parallel considerations may also apply to oysters.

Compounds capable of collapsing the transmembrane electrical potential, such as DNP, CCCP, and PCP (Smejtek *et al.*, 1976) all reduced α-MG uptake by the oyster gill. This is consistent with the gradient hypothesis. In rabbit renal brush border membrane vesicles, the Na^+ dependent transport of D-glucose is an electrogenic process and thus directly related to the transmembrane electrical-potential gradient (Beck and Sacktor, 1975). Alternatively, inhibition of α-MG accumulation by protonophores could result from inhibition of ciliary activity due to depolarization of the membrane potential (Murakami and Takahashi, 1975), and this in turn could increase the unstirred layer around the gill cells and reduce α-MG uptake.

Glucose transport by the oyster gill may depend on the transmembrane electrical potential as well as the Na^+ gradient. Compounds known to depolarize cells inhibited α-MG uptake. Naphthalene, a compound shown to hyperpolarize muscle cells (Nelson and Mangel, 1979), stimulated α-MG uptake.

The cellular metabolic rate and the rate of glucose uptake are related in many mammalian tissues (Elbrink and Bihler, 1975). This may also be true for the oyster gill when the external glucose concentration is high. In intact oysters, the rate of glucose uptake may limit the rate of irreversible disposal of glucose (net outward transport from the endogenous glucose pool) if dissolved glucose is present in the external seawater (Riley, 1981). Thus, stimulating the glucose transport system should stimulate glucose metabolism. Naphthalene stimulation of glucose metabolism has been demonstrated (Riley and Mix, 1981).

Of all the inhibitors tested, only phloridzin reduced uptake to less than that expected by diffusion alone. Alvarado (1967) demonstrated that phloridzin was a non-penetrating inhibitor which binds so as to immobilize the carrier. In the oyster gill, immobilizing the carrier reduced accumulation in both intracellular and extracellular space, thus suggesting that entry into extracellular space must be preceded by passage through intracellular space via the mobile carrier.

The conclusion that the mobile carrier is the major pathway for entry and exit of α-MG is further substantiated by the fact that in the presence of 200 μM glucose, the total leakage was 6 times greater than in the presence of 200 μM phloridzin. Leakage in the presence of 200 μM glucose represents the sum of both inner and outer leaks, as defined by Crane (1977). Leakage in the presence of 200 μM phloridzin results from only outer leaks. Inner leaks are mediated by the mobile carrier; outer leaks are not. The difference between the two treatments is a relative measure of the importance of leakage via the mobile carrier. Inner leaks are clearly much more important than outer leaks in the oyster gill.

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